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Cloning and Sequence Analysis of a Gene (*pchR*) Encoding an AraC Family Activator of Pyochelin and Ferripyochelin Receptor Synthesis in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa **K372 is deficient in the production of both the 75-kDa ferripyochelin receptor protein and pyochelin. A 1.8-kb *Eco*RI-*Sal*I fragment which restored production of both the receptor protein and pyochelin was cloned. Nucleotide sequencing of the fragment revealed an open reading frame of 888 bp, designated *pchR* (pyochelin), capable of encoding a 296-amino-acid protein of a 32,339-Da molecular mass. By using a phage T7-based expression system, a protein of ca. 32 kDa was produced off the 1.8-kb fragment, confirming that this open reading frame was indeed expressed. A region exhibiting homology to the consensus Fur-binding site of *Escherichia coli* was identified upstream of the *pchR* coding region overlapping a putative promoter. In addition, the C-terminal 80 amino acid residues of PchR showed approximately 50% homology (identity, 31%; conserved changes, 19%) to the carboxy terminus of AraC, a known transcriptional activator of gene expression in *E. coli*, *Salmonella typhimurium*, *Citrobacter freundii*, and *Erwinia chrysanthemi*. Within the C-terminal region of PchR, AraC, and a number of other members of the AraC family of transcriptional activators, there exists a highly conserved 17-residue domain where, in fact, two residues are strictly maintained and two others exhibit only conserved changes, suggesting a common functional significance to this region in all of these proteins. These data are consistent with a role for PchR as a transcriptional activator of pyochelin and ferripyochelin receptor synthesis in *P. aeruginosa*. In agreement with this, a PchR mutant obtained by in vitro mutagenesis and gene replacement was deficient in production of the ferripyochelin receptor and pyochelin.**

With perhaps few exceptions, iron is essential for the growth of bacteria. The acquisition of iron is, however, complicated by the low solubility of ferric iron in nature (50) and, for pathogenic microbes, by the iron-limiting nature of the host (59). Indeed, within humans and animals most iron is found intracellularly in, e.g., ferritin or heme (33), and that which is extracellular is bound tightly to the high-affinity iron-binding glycoproteins transferrin (in serum) and lactoferrin (in secretions) (59). In order to gain access to iron during infection, many pathogenic bacteria, therefore, synthesize and release siderophores (48). Siderophores are low-molecular-mass, high-affinity iron chelators capable of delivering iron to bacterial cells via specific receptor proteins on the cell surface (49). It is perhaps not surprising, then, that siderophores contribute to the virulence of some bacterial pathogens (20, 21, 24, 34, 55, 64).

Pseudomonas aeruginosa, an important opportunistic pathogen of humans (8) produces two known siderophores, pyoverdin (22) and pyochelin (19). Pyoverdin exhibits a much higher affinity for iron than pyochelin in vitro (66) and has been shown, also in vitro, to be capable of removing transferrin-bound iron (66). As a result, pyoverdin-producing strains of *P. aeruginosa* grow well in the presence of serum and transferrin (2). In contrast, pyochelin is less efficient at promoting growth in the presence of transferrin in vitro (3). Despite this, pyochelin production and ferripyochelin transport are important for growth and virulence of *P. aeruginosa* in vivo (21, 64).

Two receptors for ferripyochelin, of 14-kDa (65) and 75-kDa (36) molecular masses, have been identified. The gene for the 75-kDa ferripyochelin receptor has been cloned (4) and, more recently, sequenced (1). In this article, we identify and characterize a gene (*pchR*) involved in regulating the synthesis of both pyochelin and the ferripyochelin receptor protein in *P. aeruginosa*. The gene product, PchR, exhibits homology to the AraC transcriptional activator protein of enteric bacteria, consistent with a role for PchR in the iron-regulated activation of pyochelin and ferripyochelin receptor synthesis.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are described in Table 1.

Media. Iron-deficient succinate medium has been described previously (53). Amino acids (1 mM) and thiamine-HCl (30 μM) were included in growth media as required. L broth (53) was employed as the rich medium throughout. Growth media contained tetracycline (10 μg/ml), ampicillin (100 μg/ml), or kanamycin (50 μg/ml) for plasmid-bearing *Escherichia coli* and tetracycline (100 μg/ml) or carbenicillin (200 μg/ml) for plasmid-bearing *P. aeruginosa*, as necessary. Solid media were prepared by addition of Bactoagar (Difco; 1.5%, wt/vol).

Preparation of pyochelin. PAO6609 was grown overnight at 37°C in 500 ml of iron-deficient succinate medium, and pyochelin was extracted as previously described (23). Pyochelin was dissolved in 2 ml of methanol, aliquoted into 100-μl volumes, dried in an evaporator-concentrator, and stored in the dark at room temperature. Samples were resuspended, as required, in 100 μl and used immediately at

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant properties[a]	Source or reference
Strains		
P. aeruginosa		
PAO1	Prototroph	A. Kropinski
PAO6609	*met-9011 amiE200 rpsL pvd-9*	39
K372	Pch$^-$; 75-kDa ferripyochelin receptor-deficient derivative of PAO6609	36
PAO4141	*met-9020 pro-9024 blaP9202 blaJ9111 aph-9001* FP$^-$	26
CD10	PAO4141::D3112 *cts*	26
K644	PAO6609 (*pchR*::Ω-Tc)	This study
IA614	*pvd-2* Pch$^-$	5
K649	IA614::*pchR*::Ω-Tc	This study
E. coli		
JM101	*pro-lac thi* F′ (*traD36 proAB*$^+$ *lacI*^{q} ZM15)	69
K38	λ$^+$ *relA1* (HfrC)	56
S17-1	*thi pro hsdR recA* Tra$^+$	63
Plasmids		
pADD214	pUC12-derived mini-D element with RK2 origin of replication and origin of transfer; Tc^r	25
pAK1900	Multicopy *E. coli-P. aeruginosa* shuttle vector with an MCS within the *lacZ* fragment; Ap^r Cb^r	R. Sharp
pUCP18	Multicopy *E. coli-P. aeruginosa* shuttle vector; Ap^r Cb^r	62
pT7-5	pBR322 derivative carrying an MCS downstream of the strong gene 10 promoter of phage T7; Ap^r	S. Tabor
pT7-6	pT7-5 with MCS in the opposite orientation; Ap^r	S. Tabor
pGP1-2	pACYC177 derivative carrying the phage T7 RNAP gene under p_L control and the *c*I857 repressor gene; Km^r	67
pHP45-Ω-Tc	pUC18 derivative carrying an Ω fragment containing a Tc^r cartridge; Ap^r Tc^r	29
pSUP202	pBR325 derivative carrying the Mob site of plasmid RP4; Ap^r Tc^r Cam^r	63
pDH1	pADD214-derived phagemid containing 23 kb of *P. aeruginosa* chromosomal DNA; restores production of the 75-kDa ferripyochelin receptor and pyochelin in K372; Tc^r	This study
pDH2	pADD214-derived phagemid containing 26 kb of *P. aeruginosa* chromosomal DNA; restores ability to produce the 75-kDa ferripyochelin receptor and pyochelin in K372; Tc^r	This study
pDH3	pUCP18 carrying a 10.9-kb *Bam*HI-*Hin*dIII fragment derived from pDH1; restores ability to produce the 75-kDa ferripyochelin receptor and pyochelin in K372; Ap^r Cb^r	This study
pDH4	pUCP18 carrying a 7.0-kb *Eco*RI fragment derived from pDH2; restores ability to produce the 75-kDa ferripyochelin receptor and pyochelin in K372; Ap^r Cb^r	This study
pDH5	pUCP18 carrying a 2.5-kb *Eco*RI-*Kpn*I fragment derived from pDH4; Ap^r Cb^r	This study
pDH6	pUCP18 carrying a 1.8-kb *Eco*RI-*Sal*I fragment derived from pDH5; Ap^r Cb^r	This study
pDH7	pUCP18 carrying a 6.4-kb *Bam*HI fragment containing *fptA*; Ap^r Cb^r	This study

[a] Pch$^-$, pyochelin deficient; Ap^r, Cb^r, Cam^r, Km^r, and Tc^r, resistance to ampicillin, carbenicillin, chloramphenicol, kanamycin, and tetracycline, respectively; MCS, multiple cloning site; Mob, mobilization; *fptA*, the gene encoding the 75-kDa ferripyochelin receptor; RNAP, RNA polymerase.

a concentration of 0.2 μl/ml for in vivo cloning and at 100 μl/25 ml of culture for receptor induction.

Pyochelin assay. To assay for the production of pyochelin, strains of *P. aeruginosa* were cultured overnight in iron-deficient succinate medium (25 ml) at 37°C. Pyochelin was extracted from spent culture supernatants by using ethyl acetate as described elsewhere (23), resuspended in 250 μl of methanol, and chromatographed (10 μl) on Whatman thin-layer chromatography plates (Silica Gel 60 A; Chromatographic Specialties, Brockville, Ontario, Canada) with chloroform-acetic acid-ethanol (90:5:2.5) as the developing solvent. Pyochelin was visualized on thin-layer chromatography plates by using a phenolate spray reagent as previously described (23).

Outer membrane preparation and SDS-polyacrylamide gel electrophoresis. Outer membranes were obtained following differential Triton X-100 solubilization of isolated cell envelopes as previously described (61). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed as described elsewhere (45) with 9% (wt/vol) acrylamide in the running gel and without 2-mercaptoethanol in the sample loading buffer.

DNA methodology. Plasmid DNA was routinely isolated by the alkaline lysis procedure (57). For sequencing purposes, DNA was purified by using the Magic Minipreps kit (Promega Corp., Madison, Wis.) according to the manufacturer's instructions. Restriction endonucleases and T4 DNA ligase were obtained from GIBCO-BRL and used as detailed by Sambrook et al. (57). The Klenow fragment of DNA polymerase I was obtained from Pharmacia LKB Biotechnology AB (Uppsala, Sweden) and used to fill in recessed 3′ ends as described elsewhere (57). Restriction fragments were isolated, as required, from agarose gels (0.8%, wt/vol) by using the Prep-a-gene glass matrix (Bio-Rad, Mississauga, Canada) according to the manufacturer's instructions. Plasmid DNA was transformed into *E. coli* (57) and *P. aeruginosa* (7) as described previously.

In vivo cloning of *pchR*. *P. aeruginosa* chromosomal DNA was cloned by using the in vivo system of Darzins and Casadaban (25). Briefly, a lysate of *P. aeruginosa* CD10 carrying the mini-D replicon pADD214 was prepared as described previously (25) and used to infect *P. aeruginosa* K372. Following plating on iron-deficient succinate minimal media supplemented with EDDHA (53) (4 μg/ml), pyochelin (0.2 μl/ml), and tetracycline, a number of the faster-growing (larger) colonies were recovered after 16 h of growth at 37°C,

and outer membranes were prepared from these for examination on SDS-polyacrylamide gels. Those expressing the 75-kDa ferripyochelin receptor were retained for further study.

Nested deletions and nucleotide sequence determination. pDH6 contains a 1.8-kb *Eco*RI-*Sal*I fragment which restores production of pyochelin and the ferripyochelin receptor protein in K372. In order to facilitate sequencing of this region, a series of subclones containing progressively smaller portions of the 1.8-kb *Eco*RI-*Sal*I fragment was generated by using the double-stranded Nested Deletion kit (Pharmacia LKB). Briefly, the 1.8-kb *Eco*RI-*Sal*I fragment of pDH6 (Fig. 1) was blunt ended (fill-in reaction using Klenow and deoxynucleoside triphosphates) and cloned into the *Sma*I site of pAK1900 in both orientations. The two recombinant plasmids were then $CsCl_2$ purified, digested with *Sal*I and then with *Sph*I, and subjected to exonuclease III digestion according to the manufacturer's protocol. A series of nested deletions differing in size by approximately 250 to 300 bp and encompassing the length of the 1.8-kb fragment in both directions was thus generated. The deletion subclones were then purified by using the Magic Minipreps DNA purification system (Promega) and sequenced at the Centres of Excellence Core Facility for Protein/DNA Chemistry (Queen's University). The nucleotide sequences for both strands were determined, and the resulting data were analyzed by using the PC/GENE software package (Intelligenetics Inc., Mountain View, Calif.).

Expression of cloned genes in pT7 vectors. To identify protein products of cloned genes, the phage T7-based expression system of Tabor and Richardson (67) was employed. Briefly, *E. coli* K38 harboring the T7 RNA polymerase plasmid pGP1-2 and a recombinant pT7 plasmid carrying the cloned gene of interest was grown to log phase in L broth containing the appropriate antibiotics. Cells (250 μl) were harvested by centrifugation and washed twice in an iron-replete glucose minimal medium containing thiamine before being resuspended in 1 ml of this medium supplemented with 1 mM concentrations of all amino acids except cysteine and methionine. Following incubation at 37°C for 45 min, the cultures were shifted to 42°C for 30 min, at which time rifampin (400 μg/ml) was added. After a further incubation at 42°C for 15 min, the cells were pulsed with 20 μCi of [^{35}S]methionine-[^{35}S]cysteine (Expre^{35}S^{35}S; Dupont NEN, Mississauga, Canada) for 5 min at 37°C. Cells were then harvested by centrifugation, resuspended in 100 μl of gel loading buffer (45), and heated at 95°C for 5 min. The resulting whole-cell extracts were resolved on SDS-polyacrylamide gels, stained and destained briefly, dried, and exposed to Kodak X-Omat AR film (Picker International Canada, Brampton, Canada) for 16 h.

In vitro mutagenesis and gene replacement. The *pchR* gene was mutated in vitro by insertion of a tetracycline resistance gene (Ω-Tc), obtained on a 2.1-kb *Hin*dIII fragment from plasmid pHP45Ω-Tc, into the unique *Bgl*II site present within the *pchR* coding region of plasmid pDH6. The Ω-Tc-mutated gene was then recovered on a 3.8-kb *Eco*RI-*Hin*dIII fragment, which was inserted into the unique *Bam*HI site in plasmid pSUP202 (inactivating the *tet* [tetracycline resistance] gene of pSUP202). pSUP202 carrying the Ω-Tc-mutated *pchR* gene was recovered and used to transform the mobilizing *E. coli* S17-1. Plasmid-containing S17-1 was conjugated with *P. aeruginosa* PAO6609 and IA614 by pelleting equal volumes (100 μl) of donor (grown overnight at 37°C) and recipient (grown overnight at 42°C without shaking) cells in Microfuge tubes and then resuspending the cells in L broth and spotting onto the center of an L-broth plate. After incubation overnight at 37°C, bacteria on the plates were resuspended in 1 ml of L broth and plated on tetracycline-containing L-broth plates. Colonies growing up within 24 to 48 h were purified on the same medium and subsequently tested for sensitivity to carbenicillin. *P. aeruginosa* strains carrying a Ω-Tc-mutated *pchR* gene in the chromosome were tetracycline resistant but had lost the plasmid-encoded resistance to carbenicillin.

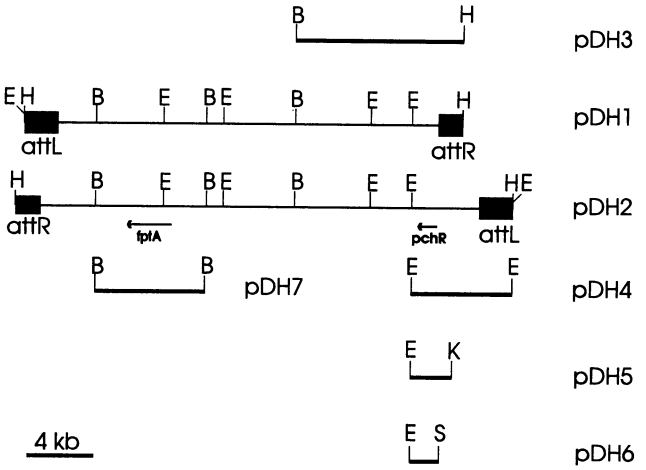

FIG. 1. Restriction map of pDH1 and pDH2. Subclones restoring production of the ferripyochelin receptor and pyochelin (pDH3 to pDH6) or ferripyochelin receptor alone (pDH7) in K372 are shown. B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; H, *Hin*dIII; S, *Sal*I. *attL* and *attR*, left and right phage arms, respectively, derived from pADD214 (26). *pchR* and *fptA* (the 75-kDa ferripyochelin receptor structural gene) are indicated.

Nucleotide sequence accession number. The nucleotide sequence is registered in the GenBank data base under accession no. L11657.

RESULTS

Cloning of genes involved in the synthesis of the ferripyochelin receptor and pyochelin. *P. aeruginosa* PAO6609, a strain deficient in the production of pyoverdin, synthesizes high levels of the 75-kDa ferripyochelin receptor protein (36) and pyochelin (35). As a result, PAO6609 grows well (forms large colonies) on iron-deficient minimal medium containing the nonmetabolizable iron chelator EDDHA (4 μg/ml) (53). A derivative of PAO6609, strain K372, lacks the receptor protein (36) and fails to synthesize pyochelin (35) and, thus, grows poorly (forms small colonies) on EDDHA-containing minimal medium, even when supplemented with pyochelin. Efforts were made, therefore, to clone the ferripyochelin receptor by restoration of good (large-colony) growth of K372 on EDDHA-pyochelin-supplemented iron-deficient minimal medium.

By using the in vivo cloning system of Darzins and Casadaban (25), two phagemid-containing K372 clones which grew more rapidly (formed larger colonies) on EDDHA-pyochelin-supplemented iron-deficient minimal medium were recovered. The phagemids (pDH1 and pDH2 [Fig. 1]) carried DNA which restored production of the 75-kDa ferripyochelin receptor to high levels (Fig. 2, lane 3) in K372. Restriction analysis of pDH1 and pDH2 revealed that a large portion (ca. 23 kb) of the cloned *P. aeruginosa*

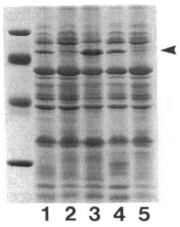

FIG. 2. SDS-polyacrylamide gel electrophoretogram of outer membranes prepared from iron-limited *P. aeruginosa* strains. Lane 1, PAO6609; lane 2, K372; lane 3, K372(pDH1); lane 4, K372(pDH6); lane 5, K644. Samples were solubilized by heating at 95°C for 5 min prior to electrophoresis. The molecular masses (in kilodaltons) of markers in the leftmost lane (from top to bottom) are 97.4 (rabbit muscle phosphorylase *b*), 66.2 (bovine serum albumin), 45.0 (hen egg white ovalbumin), and 31.0 (bovine carbonic anhydrase). Ferripyochelin receptor protein is indicated (arrowhead).

DNA was common to both phagemids (Fig. 1). An 11-kb *Bam*HI-*Hin*dIII fragment of pDH1 (pDH3 [Fig. 1]) and a 7-kb *Eco*RI fragment of pDH2 (pDH4 [Fig. 1]) were subsequently shown to restore synthesis of the ferripyochelin receptor in K372 (data not shown). These fragments shared less than 2 kb of the DNA which was subsequently recovered on a 2.5-kb *Eco*RI-*Kpn*I fragment derived from pDH4 (pDH5 [Fig. 1]). As expected, this fragment directed expression of the ferripyochelin receptor in K372 (data not shown). Finally, the DNA necessary for expression of the receptor in K372 was localized to a 1.8-kb *Eco*RI-*Sal*I fragment (pDH6 [Fig. 1 and 2, lane 4]). Unfortunately, a fragment of this size was insufficient to carry the gene encoding a 75-kDa receptor protein, indicating that we had cloned a gene involved in expression of the ferripyochelin receptor. Interestingly, pDH6 also restored synthesis of pyochelin to K372 (Fig. 3, lane 3), suggesting that the cloned gene is involved in regulating the production of pyochelin as well.

Interestingly, the levels of ferripyochelin receptor produced by K372 carrying pDH6 (or pDH3 to -5) were noticeably less than was observed initially for pDH1-containing K372 (Fig. 2, compare lanes 3 and 4). This suggests that a region of pDH1 distinct from the complementing DNA found in pDH3 to -6 contributed to maximal receptor synthesis in K372(pDH1). Indeed, a 6.4-kb *Bam*HI fragment (pDH7) was subsequently shown to direct expression of the ferripyochelin receptor, but not pyochelin, at levels comparable to that observed for pDH6 (data not shown). Fine restriction mapping and preliminary nucleotide sequencing of this region confirmed that the ferripyochelin receptor structural gene (*fptA*) was present on pDH7 (Fig. 1), which was, however, subsequently cloned (4) and sequenced (1) after

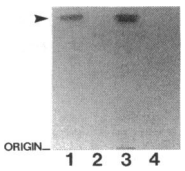

FIG. 3. Thin-layer chromatography of pyochelin extracts from iron-limited PAO6609 (lane 1), K372 (lane 2), K372/pDH6 (lane 3), and K644 (lane 4). The supernatants of overnight cultures were extracted with ethyl acetate and chromatographed on thin-layer chromatography plates. Pyochelin (arrowhead) was visualized with a phenolate spray reagent.

initiation of this study. We, therefore, focused on the 1.8-kb *Eco*RI-*Sal*I fragment.

Nucleotide sequence of the *pchR* gene. In order to identify and characterize the gene responsible for directing synthesis of pyochelin and the ferripyochelin receptor protein in K372, the 1.8-kb *Eco*RI-*Sal*I fragment of pDH6 was sequenced (Fig. 4). A large open reading frame, designated *pchR* (pyochelin) was identified on the *Eco*RI-*Sal*I fragment. *pchR* possessed two potential ATG start sites (one centered at bp 76 and the other at bp 266) both of which were preceded by sequences resembling ribosome-binding sites. By using the phage T7-based expression system of Tabor and Richardson (67), the 1.8-kb *Eco*RI-*Sal*I fragment was shown to direct synthesis of a protein with a calculated molecular mass of 32.5 kDa (Fig. 5). The deduced PchR product initiating at the second ATG (centered at bp 266) (Fig. 4) consists of 296 amino acids and has a molecular mass of 32,339 Da, in agreement with the molecular mass of the PchR product on gels and consistent with the product initiating at this position. Despite the apparently high levels of PchR produced (Fig. 5), the protein was not visible in Coomassie-stained gels (data not shown). This may be attributable to the relatively large numbers of methionine (seven) and cysteine (seven) residues in the predicted PchR product, which would provide for very efficient labeling of the protein.

Upstream of the *pchR* open reading frame, a putative promoter region was identified (Fig. 4). Interestingly, a sequence exhibiting homology to the *E. coli* Fur-binding consensus sequence (13 of 19 matches) overlaps this region (Fig. 6).

Characterization of a mutant deficient in the *pchR* product. In order to confirm that the *pchR* gene product was required for expression of pyochelin and the 75-kDa ferripyochelin receptor, a PchR$^-$ strain was constructed and examined for production of ferripyochelin receptor and pyochelin under iron-limiting conditions. As expected, the mutant, K644, was defective in ferripyochelin receptor (Fig. 2, compare lane 5 with lane 1) and pyochelin (Fig. 3, compare lane 4 with lane 1) synthesis. The 75-kDa protein seen in the K644 outer membrane appears not to be the ferripyochelin receptor, since similar levels of this protein were observed for K372 (Fig. 1) (36), which is defective in ferripyochelin uptake (36). The K644 phenotype was not the result of polar effects on essential downstream genes, since introduction of *pchR* into K644 on pDH6 restored production of pyochelin and the ferripyochelin receptor (data not shown).

Pyochelin-deficient strains of *P. aeruginosa* produce little if any ferripyochelin receptor, although the receptor is inducible in these strains in the presence of exogenously added pyochelin (32). Thus, it was possible that the influence of the *pchR* mutation on ferripyochelin receptor synthesis in K644 resulted from a lack of pyochelin necessary for induction of the receptor rather than from a direct requirement for PchR in receptor synthesis. Thus, K644 was examined for production of the ferripyochelin receptor in the presence of exogenously added pyochelin. Receptor synthesis was not induced during growth of the mutant in pyochelin-containing minimal medium (data not shown), indicating that PchR is required for receptor synthesis.

***pchR* involvement in pyochelin-mediated ferripyochelin receptor synthesis.** The observed inducibility of ferripyochelin receptor synthesis by pyochelin (32) and the failure of strain K644 to demonstrate pyochelin-inducible receptor synthesis argue that pyochelin-inducible ferripyochelin receptor synthesis is mediated by PchR. To test this directly, pyochelin-dependent induction of the ferripyochelin receptor in the

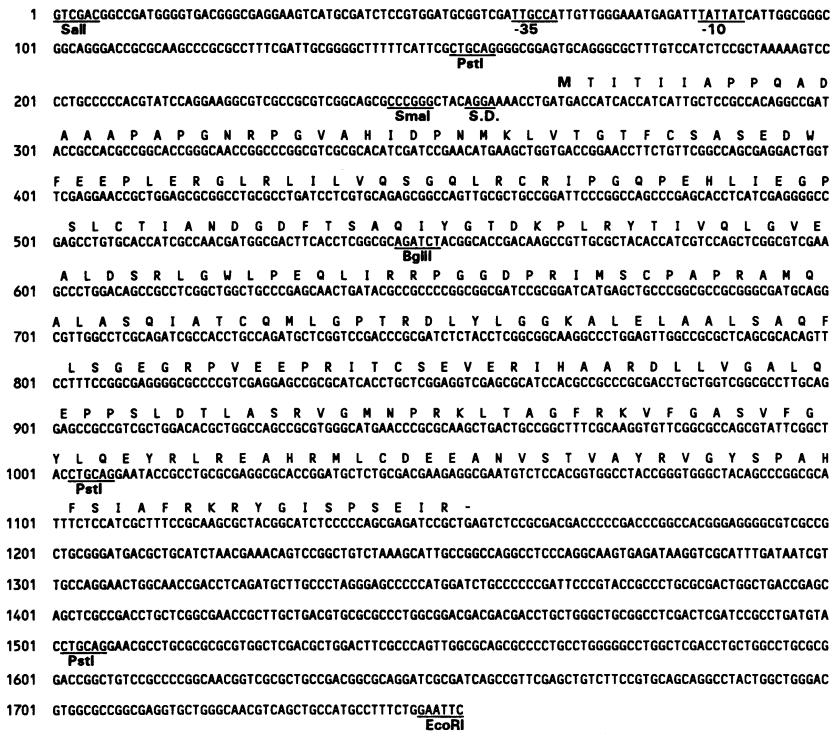

FIG. 4. Nucleotide sequence of *pchR*. The deduced amino acid sequence of PchR, putative promoter and Shine-Dalgarno sequences, and selected restriction enzyme sites are indicated.

pyochelin-deficient strain IA614 and a *pchR*::Ω-Tc derivative of this strain (K649) was examined. In agreement with the earlier results, pyochelin readily induced expression of the ferripyochelin receptor in IA614 (Fig. 7, compare lanes 1 and 2). In contrast, the receptor was not induced by pyochelin in K649 (Fig. 7, compare lanes 3 and 4), indicating that pyochelin-mediated receptor synthesis, indeed, requires the *pchR* gene product.

Homology to AraC and members of the AraC family of positive regulators. A scan of the Swiss Protein Data Base identified the AraC protein of *Salmonella typhimurium* as exhibiting homology to PchR. AraC regulates expression of the *araBAD* operon involved in enzymatically converting L-arabinose to D-xylulose 5-phosphate (60). While overall homology between AraC and PchR was low (identity, 20%; conserved changes, 11%) the C-terminal 80 amino acids of PchR showed a markedly higher degree of homology (identity, 33%; conserved changes, 17%) to AraC (Fig. 8). Two helix-turn-helix motifs which are apparently involved in DNA binding (15, 16) have been identified in this region of AraC (11). Two such motifs were identified in PchR at equivalent positions (Fig. 8). Moreover, a stretch of 17 residues at the extreme C terminus of PchR showed a high degree of similarity not only to the AraC proteins of a number of enteric organisms but also to 15 additional members of the AraC family of transcriptional regulators (Fig. 9).

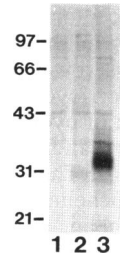

FIG. 5. Identification of PchR. The 1.8-kb *Eco*RI-*Sal*I fragment encompassing the *pchR* gene was cloned downstream of a strong phage T7 promoter in plasmids pT7-5 (lane 2) and pT7-6 (lane 3) and introduced into *E. coli* K38. Following induction of T7 RNA polymerase (see Materials and Methods), translation products of cloned genes were labeled with [^{35}S]methionine-[^{35}S]cysteine and resolved on SDS-polyacrylamide gels. Lane 1, *E. coli* K38 carrying pT7-6 without the insert. The *Eco*RI-*Sal*I fragment cloned into pT7-6 places the *pchR* gene in the same orientation as the strong T7 promoter.

DISCUSSION

P. aeruginosa K372 is a spontaneous mutant deficient in production of both pyochelin (35) and the ferripyochelin receptor (36). This phenotype is likely due to a single mutation in a gene required for expression of both pyochelin and the ferripyochelin receptor. The fact that a single gene (*pchR*) restores synthesis of both pyochelin and the ferripyochelin receptor and that a PchR mutant is defective in both receptor and pyochelin synthesis confirms this and indicates

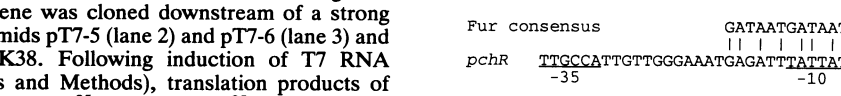

FIG. 6. Putative promoter region upstream of *pchR* in *P. aeruginosa*, showing the region of homology to the Fur-binding consensus sequence of *E. coli*. The location of this region, with respect to the initiating ATG codon of PchR, is indicated. Vertical lines represent exact matches with the consensus sequence (13).

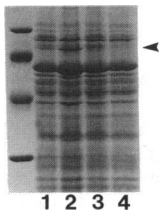

FIG. 7. Outer membrane proteins of *P. aeruginosa* IA614 (lanes 1 and 2) and K649 (lanes 3 and 4) grown in iron-deficient succinate medium without (lanes 1 and 3) or with (lanes 2 and 4) pyochelin (100 μl/25 ml of culture). The molecular masses (in kilodaltons) of markers in the leftmost lane (from top to bottom) are 97.4 (rabbit muscle phosphorylase *b*), 66.2 (bovine serum albumin), 45.0 (hen egg white ovalbumin), and 31.0 (bovine carbonic anhydrase). Ferripyochelin receptor is indicated (arrowhead).

that genes of siderophore and receptor synthesis are regulated by a common gene product.

The homology between PchR and AraC is consistent with a role for PchR in activation of expression of pyochelin and the ferripyochelin receptor. That the homology to AraC is restricted to the C-terminal regions of the proteins is significant, since it appears that, while the N terminus of AraC is involved in substrate recognition, the C terminus is specifically involved in DNA binding (15, 16, 47). In agreement with this, two potential helix-turn-helix motifs typical of DNA-binding domains have been identified in the C terminus of AraC (11), and mutations affecting AraC binding to *ara* DNA map to this region of the protein (15, 16, 30). Similarly, two potential helix-turn-helix motifs have been identified in the C terminus of PchR at positions equivalent to those found in AraC. These data suggest that, like AraC, PchR is a DNA-binding protein which activates gene expression.

AraC is representative of a family of regulatory proteins (mostly activators) which show homology at the C terminus and are predicted to operate in a similar manner. The helix-turn-helix motif appears to be a characteristic of this group of regulators and may represent a common mechanism for interacting with DNA to facilitate gene expression. Interestingly, however, the most conserved region of this family of regulators occurs near the extreme C-terminal regions of the proteins and only partially overlaps the second (in AraC and PchR) helix-turn-helix motif. Within this conserved region, two amino acids (a phenylalanine and a proline) were invariant in 20 different regulatory proteins examined. These residues are unlikely to be involved in DNA binding directly, since each regulator binds to its own unique DNA sequence. Moreover, it is charged residues which are likely to be involved in interactions (electrostatic) with DNA (71). These highly conserved residues may, however, be important for maintaining the correct disposition of the DNA-binding helix-turn-helix domains or, perhaps, for an interaction with RNA polymerase. While such an interaction has not been demonstrated, it is implied, in the case of AraC, by the overlapping of AraC and RNA polymerase binding sites in *ara* DNA and by the AraC dependence of RNA polymerase binding to *ara* DNA (43). Certainly, interactions between RNA polymerase and other activator proteins have been reported (38). Since RNA polymerase holoenzyme is likely to be highly conserved among different genera, the domains of activator proteins contacting RNA polymerase are also likely to remain relatively constant.

The promoter region identified upstream of *pchR* includes a sequence with homology (13 of 19 matches) to the consensus binding sequence for the *E. coli* Fur repressor. Since Fur is known to regulate gene expression in response to iron (9), specifically repressing expression under conditions of high concentrations of iron, this suggests that PchR expression is similarly iron regulated. This is consistent with the observed iron regulation of pyochelin (23) and the 75-kDa ferripyo-

```
PchR  MTITIIAPPQADAAAPAPGNRPGVAHIDPNMKLVTGTFCSASEDWFEEPLERGLR   55
AraC  MAETQNDPLL----------PGYSFNAHLVAGLTPIEANGYLDFFIDRPLGMKG   44

PchR  LILVQSGQLRCRIPGQPEHLIEGPSLCTIANDGDFTSAQIYGTDKPLRYTIVQLG  110
AraC  YILNLTIRGEGVINNNGEQFVCRPGDILLFPPGEIHHYGRHPDASEWYHQWVYFR   99

PchR  VEALDSRLGWLPEQLIRRPGGDPRIMSCPAPRAMQALASQIATCQMLGPTRDLYL  165
AraC  PRAYWQE--WLTWPTIFAQTGFFRPDEARQPHFSELFGQIISAGQGEGRYSELLA  152

PchR  GGKALELAALSAQFLSGEGRPVEEPRITCSEVERIHAARDLLVGALQEPPSLDTL  220
AraC  IN-------LLEQLLLRRMAVINESLHPPMDSRVRDACQYISDHLADSHFDIASV  200

PchR  ASRVGMNPRKLTAGFRKVFGASVFGYLQEYRLREAHRMLCDEEANVSTVAYRVGY  275
AraC  AQHVCLSPSRLSHLFRQQLGISVLSWREDQRISQAKLLLSTTRMPIATVGRNVGF  255

PchR  SPAH-FSIAFRKRYGISPSEIR      296
AraC  DDQLYFSRVFKKCTGASPSEFRAGCE  281
```

FIG. 8. Alignment of the deduced amino acid sequences of PchR and AraC of *S. typhimurium*. Exact matches (vertical lines) and conserved changes (dots) are indicated. Putative helix-turn-helix motifs (underlined) were found in PchR by using the PC/GENE software package. Putative helix-turn-helix motifs in AraC (16) are also indicated.

```
PchR PA   280  FSIAFRKRYGISPSEIR  296
AraC ER   266  FSRVFRKRVGVSPSDFR  282
AraC ST   261  FSRVFKKCTGASPSEFR  277
AraC EC   261  FSRVFKKCTGASPSEFR  277
AraC CF   261  FSRVFKKCTGASPSEFR  277
RhaR EC   289  FSVVFTRETGMTPSQWR  305
ToxT VC   284  FSTVFKSTMNVAPSEYL  300
TcpN VC   251  FSTVFKSTMNVAPSEYL  267
RhaS EC   254  FSTLFRREFNWSPRDIR  270
EnvY EC   228  FISVFKAFYGLTPLNYL  244
CfaD EC   243  FIRVFNKHYGVTPKQFF  259
Rns  EC   243  FIRIFNKHYGVTPKQFF  259
VirF SS   240  FIRKFNEYYGITPKKFY  256
FapR EC   233  FIKTFKEYYGVTPKKFE  249
AppY EC   212  FICAFKDYYGVTPSHYF  228
CelD EC   256  FIKTFKKLTSFTPKSYR  272
MelR EC   274  FYSTFGKYVGMSPQQYR  290
VirF YE   247  FTQSYRRRFGCTPSQAR  263
ExsA PA   271  FTQSYRRRFGCTPSRSR  287
XylS PP   297  FAENYRSAFGELPSDTL  313
                *  .  .  *
```

FIG. 9. Conserved sequence in the carboxy termini of PchR, AraC, and other AraC family transcriptional regulators. The first (left) and last (right) amino acids of the homology region in the mature sequence of each protein are indicated. Identical (*) and similar (.) amino acids are also indicated. AraC regulates expression of genes involved in L-arabinose utilization in *Erwinia chrysanthemi* (ER) (44), *S. typhimurium* (ST) (17), *E. coli* (EC) (70), and *Citrobacter freundii* (CF) (12); RhaR and RhaS regulate expression of the L-rhamnose operon in *E. coli* (68); ToxT regulates expression of virulence genes in *Vibrio cholerae* (VC) (37); TcpN regulates expression of the toxin-coregulated pilus of *V. cholerae* (51); CfaD and Rns regulate expression of CS1 and CS2 fimbrial antigens in human enterotoxigenic *E. coli* (14, 58); FapR regulates 987P fimbria expression in enterotoxigenic *E. coli*, enabling the bacteria to attach to the intestinal epithelia of piglets (42); AppY regulates expression of growth-phase-dependent gene expression in *E. coli* (6); CelD negatively regulates expression of the *cel* operon responsible for cellobiose utilization in *E. coli* (52); MelR regulates expression of the melibiose operon responsible for melibiose utilization in *E. coli* (72); VirF in *Yersinia enterocolitica* (YE) regulates expression of the virulence regulon (18); ExsA regulates expression of exoenzyme S in *P. aeruginosa* (PA) (31); XylS regulates expression of TOL plasmid genes encoding degradation enzymes of benzoate in *Pseudomonas putida* (PP) (40); VirF in *Shigella sonnei* (SS) regulates expression of the *ipa* genes involved in the invasion of epithelial cells (41); and EnvY regulates temperature-dependent expression of several envelope proteins in *E. coli* (46).

chelin receptor synthesis in *P. aeruginosa* (36). Further, it suggests that this effect of iron on expression of components of ferripyochelin transport is mediated at the level of expression of a required activator protein (PchR) rather than directly at the level of expression of structural genes for receptor and siderophore synthesis. Homology to the Fur consensus binding sequence has also been identified upstream of the structural gene for the ferric enterobactin receptor (*pfeA*) of *P. aeruginosa*, whose expression is iron regulated (27), and in fact, a Fur homolog has been identified in *P. aeruginosa* (54).

The promoter identified upstream of *pchR* is somewhat distant from the initiation codon of the gene. If it indeed functions in *pchR* expression, the resulting mRNA would possess a relatively long untranslated leader sequence. Such sequences have been identified in a number of genes, in which they appear to be involved in posttranscriptional (i.e., translational) regulation of protein synthesis (10, 28). It is interesting, therefore, that in addition to regulation by iron expression of the ferripyochelin receptor is also regulated by pyochelin. Indeed, pyochelin has been shown to specifically activate expression of the receptor protein in mutants defective in pyochelin biosynthesis (32). The demonstration here that PchR is required for this induction suggests that PchR may be regulated posttranscriptionally by pyochelin in activating receptor synthesis. If so, it is likely that PchR also upregulates pyochelin synthesis in response to the siderophore.

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